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(54) **NOVEL COMPOUND ACCELERATING SECRETION OF HUMAN-DERIVED ANTI-MICROBIAL PEPTIDE, METHOD FOR PREPARING SAME, AND COMPOSITION HAVING SAME AS ACTIVE INGREDIENT**

(57) The present invention relates to a novel compound having an acceleration effect on the secretion of human β-defensin, LL-37, which is a human-derived anti-microbial peptide, a method for preparing same, and a composition for accelerating the secretion of anti-microbial peptide having same as an active ingredient, and the compound and the composition using same of the present invention enhance the anti-microbial effect and the immunity control effect that the anti-microbial peptide has in the body by accelerating the secretion of the anti-microbial peptide in the body. The composition having the compound as an active ingredient of the present invention accelerates the secretion of innate anti-microbial peptide in the tissues and the organs in the body including the skin and can be administered through various courses including external application and oral administration, overcomes the problem of safety, the problem of resistant bacteria development, and the limitations of administration courses in a conventional anti-microbial agent, and demonstrates effective anti-microbial action.

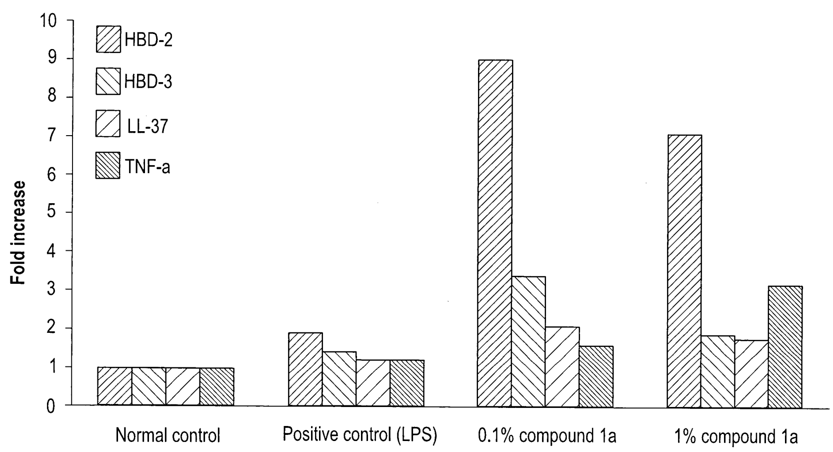


FIG. 1

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**Description**

**TECHNICAL FIELD**

**[0001]** The following disclosure relates to a compound for promoting the secretion of human antimicrobial peptides, and more particularly, to a new compound for inducing direct or indirect expression of human β -defensin-2 and -3 and LL-37, which are human antimicrobial peptides, a method for preparing the same, and a composition comprising the same as an active ingredient.

**BACKGROUND**

**[0002]** Antimicrobial peptides are natural antimicrobial materials involved in innate immunity mechanisms in *vivo,* and low-molecular weight peptide materials that retain antimicrobial activities against various microorganisms including bacteria, fungi, and viruses and induce local biophylaxis and systemic immune response. The antimicrobial peptide generally has an amphipathic structure, and according to antimicrobial mechanism thereof, a cation part thereof binds to an anionic phospholipid included in a cell membrane of the microorganism to break the cell membrane of the microorganism.

**[0003]** Defensins are one of the antimicrobial peptides that have been most studied, and are largely classified into α -defensin and β -defensin depending on structural characteristics thereof. β -defensin is a peptide material that is expressed in mucous epithelium such as skin, lungs, organs, kidneys, reproductive organs, etc. Until now, 6 sorts of human β -defensins, human β -defensin-1 (hBD-1), human β -defensin-2 (hBD-2), human β -defensin-3 (hBD-3), human β -defensin-4 (hBD-4), human β -defensin-5 (hBD-5), and human β -defensin-6 (hBD-6) have been separated and identified. In particular, while hBD-1 is uniformly expressed in epidemic cells, hBD-2, is increasingly expressed at an infected region or a physically damaged region and has been known to play an important role in controlling a systemic immune response and an inflammatory response. In addition, it has been recently reported that hBD-3 is very highly expressed at skin lesion regions of *psoriasis* patients. In recent years, it has been recently known that β -defensins are involved in not only local phylaxis but also acquired immunity resulting from chemotactic migration of dendritic cells, T lymphocytes, monocytes, etc.

**[0004]** Cathelicidins have extensive antimicrobial activities and various immunomodulatory functions. LL-37, one of the human cathelicidin degradation products, has a □-helix structure, and has extensive antimicrobial activities and inflammation modulatory functions *in vivo.* In other words, LL-37 exhibits direct antimicrobial activities against bacteria, fungi, viruses, etc., and chemotaxis for esosinophil, mononuclear cells, and T cells, and induces proliferation of endotheliocyte. In particular, LL-37 existing in the skin does prompt defense at the time of penetration of foreign antigens, and thus has antigen inhibitory functions (Braff MH, Bardan A, Nizet V, et al. Cutaneous defense mechanisms by antimicrobial peptides. J Invest Dermatol (2005) 125, 9).

**[0005]** When physical damage or infection occurs in the skin, defensin and LL-37, which are antimicrobial peptides, are secreted to induce antimicrobial activity and a systemic immune response, and particularly induce differentiation and proliferation of epidermal keratinocytes, to thereby be involved in wound healing (Niyonsaba F, Ushio H, Nakano N, et al. Antimicrobial peptides human β -defensins promote epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. J Invest Dermatol (2007) 127, 594). Also, study results have been recently reported that expression of β -defensin-2, LL-37, etc., which are antimicrobial peptides, decreased in the skin of patients with atopic dermatitis, which is a cause of high sensitivity to staphylococcus (Ong PY, et al. Endogenous Antimicrobial Peptides and Skin Infections in Atopic Dermatitis. The England Journal of Medicine (2002) 347, 1151-1160).

**[0006]** Therefore, the antimicrobial peptides play important roles in primary defense and treatment against foreign sources of infection. In particular, the antimicrobial peptides is expression-induced in the skin, thereby primarily inhibiting infection of the skin and blocking penetration of foreign sources of infection by promoting the recovery of the damaged region, and thus, play important roles in protection of skin and maintenance of skin health.

**[0007]** The related art reported materials promoting the secretion of antimicrobial peptides in *vivo.* Korean Patent Laid-Open Publication No. 10-2006-0076775 discloses that various organic acids promote β -defensin secretion and International Patent Laid-Open Publication No. WO 0168085 discloses that amino acid, isoleucine, promotes defensin secretion. However, there are limitations in that some materials have unfavorable effects and other materials induce simultaneous secretion of inflammatory cytokines as well as antimicrobial peptides. For this reason, there have been demands for compounds having superior activity of promoting secretion of antimicrobial peptide and not inducing secretion of inflammatory cytokines.

**[0008]** The present inventors synthesized various materials in order to produce new materials for promoting antimicrobial peptides for a long time, and conducted experiments for activities thereof. As a result, the present inventors synthesized new compounds having excellent effects in promoting the secretion of human β - defensins and LL-37 *in vivo*, and completed the present invention.

## SUMMARY

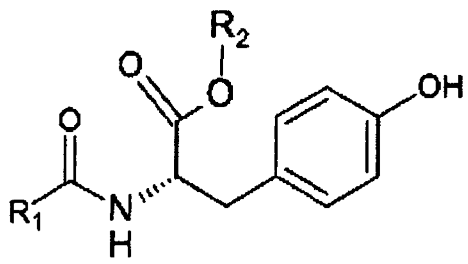
**[0009]** An embodiment of the present invention is directed to providing a new compound for promoting secretion of human antimicrobial peptide *in vivo.*

**[0010]** Another embodiment of the present invention is directed to providing a method for preparing the new compound for promoting secretion of human antimicrobial peptides *in vivo.*

**[0011]** Still another embodiment of the present invention is directed to providing a composition including the new compound for promoting secretion of human antimicrobial peptides in *vivo* as an active ingredient.

**[0012]** In one general aspect, there is provided a new compound represented by Chemical Formula (I) below:

**[0013]**

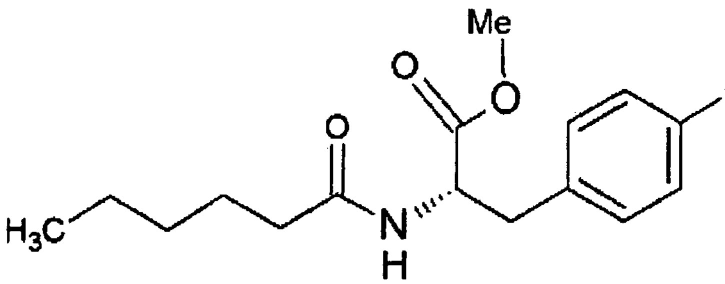



(I)

**[0014]** (wherein, $R_1$ is C1~C17 straight chain and branched alkyl, phenyl, or benzyl; and $R_2$ is hydrogen, methyl, or ethyl).

**[0015]** The compound of Chemical Formula may have an effect of promoting secretion of human antimicrobial peptides, specifically, β -defensin and/or LL-37. Here, a more preferable compound may be a compound where $R_1$ is C5 straight chain alkyl and $R_2$ is methyl in the chemical formula above, and may be represented by Chemical Formula (Ia) below.

**[0016]**




(Ia)

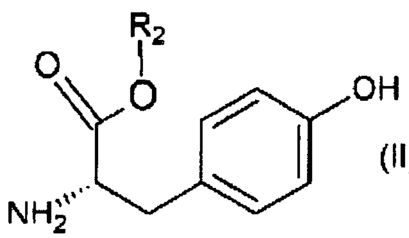
**[0017]** In another general aspect, there is provided a method for preparing a new compound for promoting secretion antimicrobial peptide *in vivo,* including:

**[0018]** (A) dissolving a compound of Chemical Formula (II) or hydrochloride thereof in an organic solvent in the presence of organic base; and

**[0019]** (B) adding a compound of Chemical Formula (III) thereto at a reaction temperature of 0°C~5°C, followed by stirring.

**[0020]** <u>Compound of Chemical Formula (II)</u>

**[0021]**

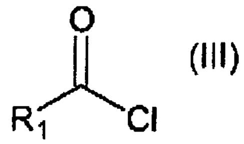



(II)

**[0022]** [wherein, $R_2$ is hydrogen, methyl, or ethyl]

**[0023]** Compound of Chemical Formula (III)

**[0024]**



**[0025]** [wherein, $R_1$ is C1~C17 straight chain and branched alkyl, phenyl, or benzyl]

**[0026]** In still another general aspect, there is provided a composition for promoting secretion of antimicrobial peptides *in vivo,* the composition including the new compound of Chemical Formula (I) as an active ingredient.

**[0027]** Here, as the active ingredient, the compound of Chemical Formula (I) or Chemical Formula (Ia) may be contained at 0.001~90 wt%, and more preferably 0.001~50 wt%, in the composition.

**[0028]** Here, the formulation type of the composition may not be particularly limited as long as it is applicable for the human body including mucous membranes, such as, skin, respiratory tract, oral cavity, nasal cavity, or the like, as a local administration agent, and the composition may be prepared in a liquid phase, an emulsion phase, a suspension phase, a cream phase, an ointment phase, a gel phase, a jelly phase, or a spray phase.

**[0029]** Here, the formulation type of the composition may not be particularly limited as long as it is applicable for the human body as a systemic administration, and the composition may be prepared as an oral administration, an injection, or the like.

**ADVANTAGEOUS EFFECTS**

**[0030]** As set forth above, since the human antimicrobial peptides, defensin and LL-37, have prompt antimicrobial activities and are secreted in various tissues and organs of the body, including skin, the new compound of the present invention for promoting secretion of defensin and LL-37 and the composition including the same were administered for external use or for internal use, thereby exhibiting effective antimicrobial activities on various regions of the body while overcoming restricted administration paths, generation of antibiotic bacteria, safety problems, and the like, of the existing antimicrobial agents.

**[0031]** The material for promoting secretion of human defensin and LL-37 in *vivo* of the present invention can induce the promotion of secretion of β -defensin-2 and LL-37 in *vivo* at the applied region thereof, and thus enhance antimicrobial activities and immune functions, thereby improving bio-defense response. Therefore, the material for promoting secretion of antimicrobial peptides of the present invention is effective in prediction and treatment of syndromes resulting from infection by foreign antigens, such as bacteria, fungi, and viruses. In particular, the present invention induces an increase in antimicrobial peptides at a lesion region of atopic dermatitis where the antimicrobial peptides are reduced, and thus can obtain effective advantages in suppressing infection of the skin.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**[0032]** FIG. 1 is a graph showing expression degrees of human antimicrobial peptides, hBD-2 and LL-37 in *vivo*, by a compound of the present invention;

**[0033]** FIG. 2 is a graph showing skin barrier recovery capability in the mouse coated with the compound of the present invention;

**[0034]** FIG. 3 is a graph showing anti-inflammatory efficacy of the compound of the present invention in an animal model with atopic dermatitis;

**[0035]** FIG. 4 is an image showing expression degrees of mouse antimicrobial peptide, CRAMP, which has a similar structure to human antimicrobial peptide, LL-37, in the corneum of the skin, when the compound of the present invention is coated on an atopic animal model;

**[0036]** FIG. 5 is an image showing proliferation degrees of cells in the epidemic layer when the compound of the present invention is coated on an atopic animal model;

**[0037]** FIG. 6 is a graph showing mast cells when the compound of the present invention is coated on an atopic animal model; and

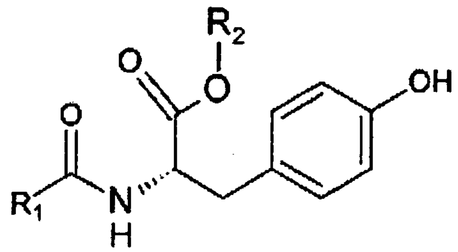
**[0038]** FIG. 7 is a graph showing cytotoxicity of the compound of the present invention.

**DETAILED DESCRIPTION OF EMBODIMENTS**

**[0039]** Hereinafter, a new compound for promoting secretion of human antimicrobial peptides *in vivo* according to the present invention will be described in detail.

**[0040]** A new compound for promoting secretion of human antimicrobial peptides *in vivo* has a structure of Chemical Formula (I) below:

**[0041]**

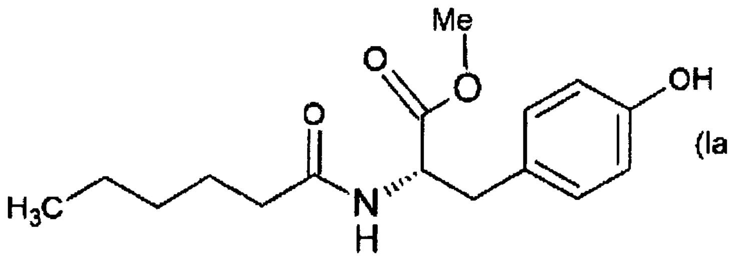


(I)

**[0042]** (wherein, $R_1$ is C1~C17 straight chain and branched alkyl, phenyl, or benzyl; and $R_2$ is hydrogen, methyl, or ethyl).

**[0043]** In the present invention, a compound where $R_1$ is C5 straight chain alkyl and $R_2$ is methyl in the chemical formula above, is more preferable, and represented by Chemical Formula (Ia) below:

**[0044]**



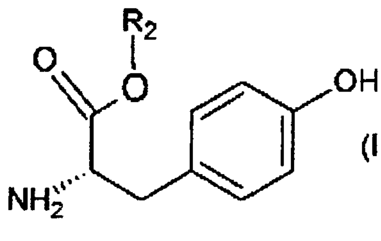
(Ia)

**[0045]** The compound of Chemical Formula (I) is prepared by the following method.

**[0046]** The compound of Chemical Formula (I) may be prepared by including: dissolving a compound of Chemical Formula (II) or hydrochloride thereof in an organic solvent in the presence of organic base; adding a compound of Chemical Formula (III) thereto at a reaction temperature of 0°C~5°C, followed by stirring; and extracting, drying, and filtering the reacted material.

**[0047]** Compound of Chemical Formula (II)

**[0048]**

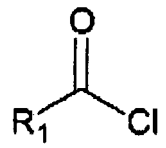


(II)

**[0049]** [Wherein, $R_2$ is hydrogen, methyl, or ethyl.]

**[0050]** Compound of Chemical Formula (III)

**[0051]**



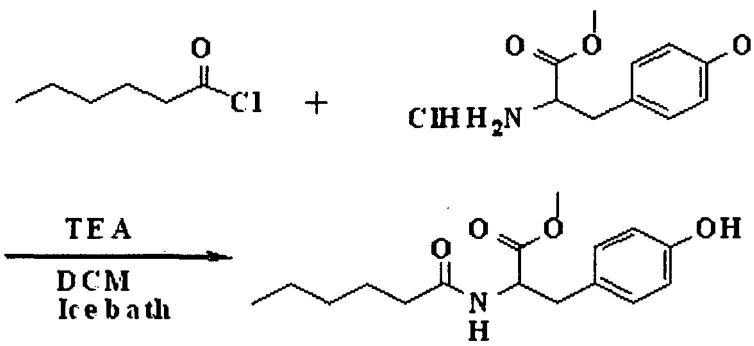
(III)

**[0052]** [Wherein, $R_1$ is C1~C17 straight chain and branched alkyl, phenyl, or benzyl.]

**[0053]** In the above reaction, the organic base is preferably selected from the group consisting of triethyl amine, diethyl amine, trimethyl amine, and dimethyl amine, and more preferable is trimethyl amine. In addition, the organic solvent used in the reaction is preferably selected from the group consisting of dichloro methane, N,N-dimethyl formamide, chloroform, acetonitrile, and acetone, and more preferable is dichloromethane. In the reaction, the step (B) is preferably carried out by stirring for 3~4 hours at 0°C.

**[0054]** The compound of Chemical Formula (Ia) as a representative compound of the compound of Chemical Formula (I) may be prepared as shown in the Reaction Scheme below.

**[0055]**




(Ia)

**[0056]** Dichloro methane (DCM) is added to (L)-tyrosine methyl ester HCl salt, and triethyl amine (TEA) is added thereto, thereby dissolving HCl salt. The reactor is temperature-lowered to 0°C by using an ice bath, and hexanoyl chloride is slowly added, following by stirring for 3-4 hours. Water is added to the reacted material, followed by extraction with ethyl acetate. Drying over sodium sulfate followed by filtering is carried out. The solvent is removed from the filtrate by rotary evaporation, followed by solidification with hexane and ethyl acetate, thereby obtaining a white solid [Compound of Chemical Formula (Ia)].

**[0057]** As the experiment results confirming whether the thus synthesized compound of Chemical Formula (Ia) simulates secretion of β -defensin and cathelicidin, it can be seen that the compound of the present invention promotes secretion of β -defensin-2 and LL-37 in human cells that are cultured.

**[0058]** In addition, it can be confirmed that, by using the compound of Chemical Formula (Ia), expression of antimicrobial peptides is increased in the skin and anti-inflammatory efficacy and skin barrier function are improved as the result of application to an animal model with atopic dermatitis, and skin barrier recovery is significantly promoted as the result of experiments using an animal model with acute skin barrier disruption.

**[0059]** [Examples]

**[0060]** Hereinafter, the present invention will be described through the following examples and experimental examples, but the present invention is not limited to these examples.

**[0061]** **Example 1**

**[0062]** **Preparation of (*S*)-Methyl 2- (hexanamido) -3- (4-hydroxyphenyl)propanoate**

**[0063]** 200 ml of dichloro methane (DCM) was added to (L)-tyrosine methyl ester HCl salt (23.16g, O.lmol), and 28 ml of triethyl amine (TEA, 0.2 mol) was added thereto, thereby dissolving HCl salt. The reactor was temperature-lowered to 0°C by using an ice bath, and hexanoyl chloride (13.8ml, 0.1mol) was slowly added, following by stirring for 3~4 hours. 200 ml of water was added to the reacted material, followed by extraction with 400 ml of ethyl acetate. Drying over sodium sulfate followed by filtering was carried out. The solvent was removed from the filtrate by rotary evaporation, followed by solidification with hexane and ethyl acetate, thereby obtaining a white solid (27.1g, 92.5% yield) [hereinafter, referred to as Compound 1a].

**[0064]** mp : 96°C

**[0065]** NMR (400MHz $CDCl_3$) $^1H$ : 0.873 (3H, t, $\underline{CH_3}CH_2$), 1.261(4H, m, $CH_3\underline{CH_2CH_2}$), 1.584 (2H, m, $CH_2CH_2\underline{CH_2}CH_2CO$), 2.175(2H, t, $CH_2\underline{CH_2}CO$), 2.955~3.118(2H, dd,dd, $CH\underline{CH_2}Ph$), 3.742(3H, s, $OCH_3$), 4.883(1H, m, $CH_2\underline{CH}(NH)CO$), 5.922 (1H, d, NH), 6.720(2H, d, $\underline{CH}C(OH)\underline{CH}$), 6.933(2H, d, $\underline{CH}(CH_2)C\underline{CH}$)

**[0066]** **Example 2**

**[0067] Preparation of (S)-Methyl 3-(4-hydroxyphenyl)-2-(octanamido)propanoate**

**[0068]** 200 ml of dichloro methane (DCM) was added to (L)-tyrosine methyl ester HCl salt (23.16g, O.lmol), and 28 ml of triethyl amine (TEA, 0.2 mol) was added thereto, thereby dissolving HCl salt. The reactor was temperature-lowered to 0°C by using an ice bath, and 16.6 ml of octanoyl chloride (O.lmol) was slowly added, following by stirring for 3~4 hours. 200 ml of water was added to the reacted material, followed by extraction with 400 ml of ethyl acetate. Drying over sodium sulfate followed by filtering was carried out. The solvent was removed from the filtrate by rotary evaporation, followed by separation by column chromatograph using hexane and ethyl acetate (Hex:EA=2:1), thereby obtaining a white solid (30.1g, 93.3% yield).

**[0069]** mp : 76°C

**[0070]** NMR (400MHz $CDCl_3$) $^1H$ : 0.881(3H, t, $\underline{CH_3}CH_2$), 1.262(8H, m, $CH_3\underline{CH_2CH_2CH_2CH_2}$), 1.581 (2H, m, $CH_2\underline{CH_2}CH_2CO$), 2.177(2H, t, $CH_2\underline{CH_2}CO$), 2.960~3.111(2H, dd,dd, $CH\underline{CH_2}Ph$), 3.740(3H, s, $\underline{OCH_3}$), 4.888(1H, m, $CH_2\underline{CH}(NH)CO$), 5.926 (1H, d, NH), 6.725(2H, d, $\underline{CH}C(OH)\underline{CH}$), 6.931(2H, d, $\underline{CH}(CH_2)C\underline{CH}$)

**[0071] Example 3**

**[0072] Preparation of (S)-Methyl 2-(dodecanamido)-3-(4-hydroxyphenyl)propanoate**

**[0073]** 300ml of dichloro methane (DCM) was added to (L)-tyrosine methyl ester HCl salt (23.16g, O.lmol) and 28 ml of triethyl amine (TEA, 0.2 mol) was added thereto, thereby dissolving HCl salt. The reactor was temperature-lowered to 0°C by using an ice bath, and 21.88g of dodecanoyl chloride (O.lmol) was slowly added, following by stirring for 3~4 hours. 200 ml of water was added to the reacted material, followed by extraction with 500ml of ethyl acetate. Drying over sodium sulfate followed by filtering was carried out. The solvent was removed from the filtrate by rotary evaporation, followed by separation by column chromatograph using hexane and ethyl acetate (Hex:EA=2:1), thereby obtaining a white solid (34.2g, 90.5% yield).

**[0074]** mp : 89°C

**[0075]** NMR (400MHz $CDCl_3$) $^1H$ : 0.881 (3H, t, $\underline{CH_3}CH_2$), 1.262(16H, m, $CH_3\,\underline{(CH_2)_8}$), 1.581(2H, m, $CH_2\underline{CH_2}CH_2CO$), 2.177(2H, t, $CH_2\underline{CH_2}CO$), 2.960~3.111(2H, dd,dd, $CH\underline{CH_2}Ph$), 3.740(3H, s, $\underline{OCH_3}$), 4.888(1H, m, $CH_2\underline{CH}(NH)\,CO$), 5.926 (1H, d, NH), 6.725(2H, d, $\underline{CH}C(OH)\underline{CH}$), 6.931(2H, d, $\underline{CH}(CH_2)C\underline{CH}$)

**[0076] Example 4**

**[0077] Preparation of (S)-Methyl 3-(4-hydroxyphenyl)-2-(palmitamido)propanoate**

**[0078]** 300ml of dichloro methane (DCM) was added to (L)-tyrosine methyl ester HCl salt (23.16g, 0.1mol), and 28 ml of triethyl amine (TEA, 0.2 mol) was added thereto, thereby dissolving HCl salt. The reactor was temperature-lowered to 0°C by using an ice bath, and 27.49g of palmitoyl chloride (O.lmol) was slowly added, following by stirring for 3~4 hours. 200 ml of water was added to the reacted material, followed by extraction with 500ml of ethyl acetate. Drying over sodium sulfate followed by filtering was carried out. The solvent was removed from the filtrate by rotary evaporation, followed by solidification with hexane and ethyl acetate, thereby obtaining a white solid (39.4g, 90.8% yield).

**[0079]** mp : 100°C

**[0080]** NMR (400MHz $CDCl_3$) $^1H$ : 0.881(3H, t, $\underline{CH_3}CH_2$), 1.262(24H, m, $CH_3\underline{(CH_2)_{12}}$), 1.581(2H, m, $CH_2\underline{CH_2}CH_2CO$), 2.177(2H, t, $CH_2\underline{CH_2}CO$), 2.960~3.111(2H, dd,dd, $CH\underline{CH_2}Ph$), 3.740(3H, s, $\underline{OCH_3}$), 4.888(1H, m, $CH_2\underline{CH}(NH)CO$), 5.926 (1H, d, NH), 6.725(2H, d, $\underline{CH}C(OH)\underline{CH}$), 6.931(2H, d, $\underline{CH}(CH_2)C\underline{CH}$)

**[0081] Example 5**

**[0082] Preparation of (S)-Methyl 3-(4-hydroxyphenyl)-2-(phenylacetamido)propanoate**

**[0083]** 200ml of dichloro methane (DCM) was added to (L)-tyrosine methyl ester HCl salt (23.16g, O.lmol), and 28 ml of triethyl amine (TEA, 0.2 mol) was added thereto, thereby dissolving HCl salt. The reactor was temperature-lowered to 0°C by using an ice bath, and 15.46g of phenylacetyl chloride (O.lmol) was slowly added, following by stirring for 3~4 hours. 200 ml of water was added to the reacted material, followed by extraction with 400 ml of ethyl acetate. Drying over sodium sulfate followed by filtering was carried out. The solvent was removed from the filtrate by rotary evaporation, followed by solidification with hexane and ethyl acetate, thereby obtaining a white solid (27.7g, 88.1% yield).

**[0084]** mp : 101°C

**[0085]** NMR (400MHz $CDCl_3$) $^1H$ : 2.852~3.032(2H, dd,dd, $CH\underline{CH_2}Ph$), 3.554(2H, s, $Ph\underline{CH_2}CO$) 3.709(3H, s, $\underline{OCH_3}$), 4.837(1H, m, $CH_2CH(NH)CO$), 5.917(1H, d, NH), 6.628(2H, d, $\underline{CH}C(OH)\underline{CH}$), 6.724(2H, d, $\underline{CH}(CH_2)C\underline{CH}$), 7.155~7.348 (5H, m, $\underline{Ph}CH_2$)

**[0086] Example 6: Production of Cream** Formulation

**[0087]** A cream formulation having the following composition was produced by a general method for forming cream.

**[0088]**

Table 1

| | Function | Ingredient | Weight (%) |
|---|---|---|---|
| Aqueous Phase | Antiseptic | Methyl paraben | 0.2 |

(continued)

| | Function | Ingredient | Weight (%) |
|---|---|---|---|
| | Polymer | xanthan gum | 0.1 |
| | Moisturizer | Glycerine | 8.0 |
| | Purified Water | Water | 74.0 |
| Oil Phase | Fatty Acid | Stearic acid | 2.0 |
| | Higher Alcohol | Cetanol | 2.0 |
| | Wax | Bees wax | 2.0 |
| | Surfactant | POE(15) GMS | 2.5 |
| | Surfactant | POE(10) GMS | 1.0 |
| | Surfactant | GMS | 1.5 |
| | Oil | Macademia nut oil | 3.0 |
| | Oil | Squalane | 3.0 |
| | Antiseptic | Propyl paraben | 0.1 |
| | Active ingredient | Synthesized Material of Example 1 (Compound 1a) | 0.5 |
| Additive | Spice | Fragrance | 0.1 |

**[0089]** It was confirmed that cream produced from Example 6 had excellent storage stability and feeling of use.

**[0090]** In order to assess promotion of antimicrobial peptide secretion by using Compound 1a of Example 1, in vitro experiments were conducted.

**[0091]** **Test Example 1: Promotion of Secretion of β-defensin and LL-37**

**[0092]** A medium containing 1% penicillin/streptomycin but not serum was used to culture human keratinocyte (HaCaT). The human keratinocyte was cultured in a 5% $CO_2$ incubator at 37°C. The cells were seeded in each well of a 6-well plate at $3\times10^5$ cells/well, and then cultured for 48 hours. 1.7 mM calcium chloride and the new material synthesized from Example 1 were added thereto, and the cells were allowed to culture for 24 hours.

**[0093]** For assessment, at least one untreated control and at least one positive control were used together. 2.5ng/mL of lipopolysaccharides (LPS), which is known to promote expression of hBD-2 and LL-37, was used as the positive control, and was allowed to react. After the reaction was completed, a supernatant was collected, and then the cells were washed with phosphate buffer saline (PBS) and collected by using a trypsin-EDTA solution, and stored in a tube. 1 ml of a triazole reagent was added to extract mRNA. After reaction for 15 seconds at room temperature, 200 $\mu\ell$ of chloroform was added. Centrifugal separation at 13,000 rpm was carried out for 10 minutes. The supernatant was transferred into another tube, and then 500$\mu\ell$ of isopropanol was added thereto, followed by centrifugal separation at 13,000 rpm for 10 minutes. The precipitated RNA wash washed by using 70% ethanol. After washing was carried out two times, RNA was dissolved by using distilled water at the time of the third washing. The diluted RNA was analyzed at a wavelength between 260nm and 280nm, and quantitated.

**[0094]** The thus obtained RNA was subjected to RT-PCR procedure to obtain PCR results. For the RT-PCR, 2$\mu\ell$ of $MgCl_2$, 1$\mu\ell$ of RT buffer, 1$\mu\ell$ of dNTP mix, 0.25$\mu\ell$ of Rnase inhibitor, 0.5$\mu\ell$ of RTase, 0.5$\mu\ell$ of oligo dT, 3.75$\mu\ell$ of distilled water, and 2$\mu$g of RNA were placed in the tube, and then were allowed to react. The RT-PCR conditions were 45°C for 1 hour and 95°C for 5 minutes. PCR was carried out for qualitative analysis on GAPDH, hBD-2 and -3, and LL-37. The used primers were obtained from the following documents (Kim JE, Kim BJ, Jeong MS, et al, Expression and Modulation of LL-37 in Normal Human Keratinocytes HaCaT Cells and Inflammatory Skin Diseases. J Korean Med Sci (2005) 20, 649; Pernet I, Reymermier C, Guezennec A, et al, Calcium triggers beta-defensin (hBD-2 and hBD-3) and chemokine macrophage inflammatory protein-3 alpha (MIP-3alpha/CCL20) expression in monolayers of activated human keratinocytes. Exp Dermatol (2003) 12, 755).

**[0095]** The used primers are as follows.

**[0096]** GAPDH sense: 5'- GGG CAT GAA CCA TGA GAA GT -3'

**[0097]** GAPDH antisense: 5'-GTC TTC TGG GTG GCA GTG AT -3'

**[0098]** hBD-2 sense: 5'- CCA GCC ATC AGC CAT GAG GGT-3'

**[0099]** hBD-2 antisense: 5'-GGA GCC CTT TCT GAA TCC GCA-3'

**[0100]** hBD-3 sense: 5'-TTC CAG GTC ATG GAG GAA TC -3'

**[0101]** hBD-3 antisense: 5'-GAG CAC TTG CCG ATC TGT TC -3'

**[0102]** TNF-α sense: 5'- GAG AAG GGT GAC CGA CTC AG -3'

**[0103]** TNF-α antisense: 5'- ATG TTC GTC CTC CTC ACA GG -3'

**[0104]** LL-37 sense: 5'-TCG GAT GCT AAC CTC TAC CG-3'

**[0105]** LL-37 antisense: 5'-GGG TAC AAG ATT CCG CAA AA-3'

**[0106]** 12.5μℓ of PCR premix, 2μℓ of primer sense (10μM), 2μℓ of primer antisense (10μM),1.5μℓ of cDNA, and 7μℓ of distilled water were inputted, and then PCR was carried out. PCR conditions of hBD-2 and -3 were 30 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds, and then 72°C for 10 minutes. PCR conditions of LL-37 were 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and then 72°C for 10 minutes. After amplification, the final products were mixed together, the final solution was loaded on an agaros gel containing a nucleic acid insertion visible under UV (such as, ethidium bromide), which is gelled at 1.5%. The sample was migrated and then read out under UV in a dark room, and digitally photographed. Photos of the gel were analyzed by image processing software which quantifies the band intensities. As basal levels of defensin and LL-37 expression (untreated control) were not detectable, antimicrobial peptide expression was detectable by using intensity ratios of the hBD-2/GAPDH, hBD-3/GAPDH, and LL-37/GAPDH bands in the positive control and sample-treated groups. Real-time PCR was carried out in order to more clarify these results. 5μℓ of Sybergreen, 2μℓ of primer sense (10uM), 2μℓ of primer antisense (10μM),1μℓ of cDNA, and 1μℓ of distilled water were inputted, and then the real-time PCR was carried out.

**[0107]** The results are shown in FIG. 1. As can be seen in FIG. 1, it can be seen that the compound of the present invention promotes the secretion of human β-defensin-2 and LL-37 from cultured human-derived cells.

**[0108]** **Test Example 2: Recovery of Skin Barrier damage**

**[0109]** Skin barriers of nude mice were acutely damaged, and then effects of the new material represented in Example 1 on recovery of acute skin barriers were assessed. D-Squame was used to induce skin barrier damage to left and right back regions of 6-8 week aged nude mice. Here, all the experimental groups were maintained to have no difference in trans epidermal water loss (TEWL) through measurement of the trans epidermal water loss (TEWL), and then vehicle (PEG:EtOH=7:3) and the new material represented in the example were coated thereon. The trans epidermal water loss (TEWL) was measured at the time of 0h, 3h, 6h, and 24h to confirm recovery of the skin barrier, and skin biopsies were conducted at the respective time, to thereby implement histological examination and other examinations.

**[0110]** The results are shown in FIG. 2. It can be confirmed from the results of FIG. 2 that the new compound of the present invention show significant results on the recovery of skin barrier damage.

**[0111]** **Test Example 3: Efficacy Assessment on Animal Model with Atopic** Dermatitis

**[0112]** An animal model with atopic dermatitis was constructed by coating the abdomens of 6-8 week aged nude mice with 5% oxazolone solution one time for percutaneous sensitization; after week, with 0.1% oxazolone solution every other day, six times, and 1% oxazolone solution every other day, four times. It has been reported that in the case of the animal model with atopic dermatitis using oxazolone, antimicrobial peptides were reduced in the skin (Man M-Q, Hatano Y, Lee SH, et al. Characterization of a hepten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges. J Invest Dermatol (2008) 128, 79-86). The skin of the animal model with atopic dermatitis constructed as above was coated with vehicle (PEG: EtOH=7:3) and Compound Ia of Example 1 diluted with the vehicle at a concentration of 0.1% once in the morning and in the afternoon for a total of four days. On the last day, thickness of the skin was measured. Corticosteroid based drug, Dexamethasone, which is an effective anti-inflammatory agent, was used as a positive control.

**[0113]** The obtained results are shown in FIG. 3. These results confirmed that the new material synthesized according to the example had an inflammatory effect in the animal model with atopic dermatitis.

**[0114]** The new compound synthesized according to Example 1 was coated for four days, and then skin tissue biopsy was conducted to form a paraffin block. The tissue was allowed to adhere on the slide by using a paraffin cutter. After 500μℓ of a peroxides blocking reagent was loaded, the reaction was carried out for 30 minutes. Washing with PBS solvent was carried out three times at a time interval of 5 minutes. After 500μℓ of a peroxide blocking reagent was loaded, the reaction was carried out for 15 minutes. The first goat anti-mouse CRAMP was allowed to react at 25°C for 30 minutes. The reaction using donkey anti-goat IgG-HRP as an antibody was carried out at 25°C for 30 minutes. The reaction using DAB as a color forming agent was carried out for 10 minutes. After the reaction was completed, expression of CRAMP in the corneum of the skin was measured through a microscope. The obtained results were tabulated in Table 4. A), B), C), and D) of FIG. 4 show Normal, Negative control, 0.01% Dexamethasone, and 0.1% Compound 1a, respectively.

**[0115]** Another slide was used to measure proliferating cell nuclear antigen (PCNA). After 500μℓ of a peroxides blocking reagent was loaded, the reaction was carried out for 30 minutes. Washing with PBS solvent was carried out three times at a time interval of 5 minutes. After 500μℓ of a peroxides blocking reagent was loaded, the reaction was carried out for 15 minutes. The first rabbit anti-mouse PCNA was allowed to react out at 25°C for 30 minutes. The reaction using donkey anti-rabbit IgG-HRP as an antibody was carried out at 25°C for 30 minutes. The reaction using DAB as a color forming agent was carried out for 10 minutes. After the reaction was completed, proliferation of skin cells in the epidermis layer of the skin was measured through a microscope. The obtained results are shown in FIG. 5.

**[0116]** Another slide was used to measure mast cells. After paraffin was removed from the slide, the reaction in the potassium permanganate solution was carried out for 2 minutes. The slide was washed by using distilled water. The reaction in the potassium metabisulphite solution was carried out for 1 minute. The slide was washed by using distilled water for 3 minutes. The reaction in the acidified toluidine blue solution was carried out for 5 minutes. The slide was washed by using distilled water. After the reaction was completed, mast cells of the skin were measured through a microscope. The obtained results are shown in FIG. 6.

**[0117]** It can be seen from the above test results that the new compound of the present invention has superior inflammatory efficacy and significantly promotes improvement and recovery of skin barrier.

**[0118]** **<u>Test Example 4: Cytotoxicity</u>**

**[0119]** A medium containing 1% penicillin/streptomycin but not serum was used to culture human keratinocyte (HaCaT). The human keratinocyte was cultured in a 5% CO2 incubator at 37°C. The cells were seeded in each well of a 96-well plate at 2.5x $10^4$ cells/well, and then cultured for 48 hours. The cells were further cultured for 24 hours by using a medium without serum. On the next day, the new material synthesized by the example was added at different concentrations thereof, and then the reaction was carried out for 24 hours. After four hours after the color forming reagent was put, apoptosis was measured at an absorbance of 590nm. The obtained results are shown in FIG. 7.

**[0120]** It can be seen from the above test results that the new compound of the present invention has also no problems in view of stability.

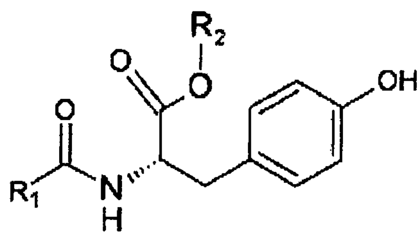
## INDUSTRIAL APPLICABILITY

**[0121]** The present invention is directed to an industrially applicable novel compound capable of inducing direct or indirect expression of human β -defensin-2 and -3 and LL-37, which are human antimicrobial peptides, a method for preparing the same, and a composition comprising the same as an active ingredient.

**[0122]**

## Claims

1. A new compound for promoting secretion of human antimicrobial peptides *in vivo*, the new compound represented by Chemical Formula (I) below:

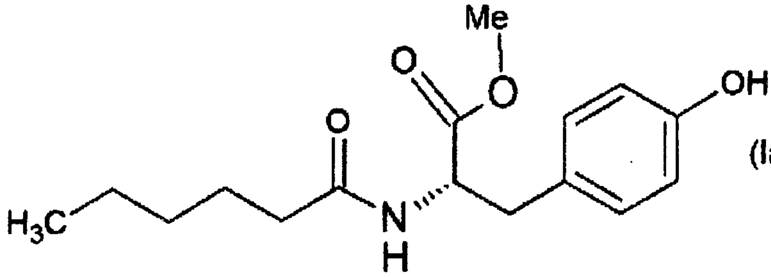



(I)

(wherein, $R_1$ is C1~C17 straight chain and branched alkyl, phenyl, or benzyl; and $R_2$ is hydrogen, methyl, or ethyl).

2. The new compound of claim 1, wherein the antimicrobial peptides are human β -defensin-2, β - defensin-3, and LL-37.

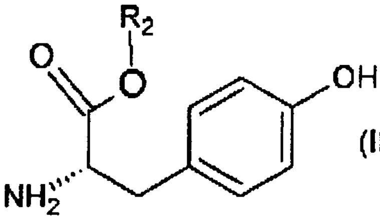
3. The new compound of claim 1, wherein the new compound is a compound where $R_1$ is C5 straight chain alkyl and $R_2$ is methyl in Chemical Formula (I), and represented by Chemical Formula (Ia):



(Ia)

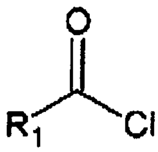
4. A method for preparing a new compound for promoting secretion antimicrobial peptides *in vivo,* the method comprising:

(A) dissolving a compound of Chemical Formula (II) or hydrochloride thereof in an organic solvent in the presence of organic base; and
(B) adding a compound of Chemical Formula (III) thereto at a reaction temperature of 0°C~5°C, followed by stirring:



(II)

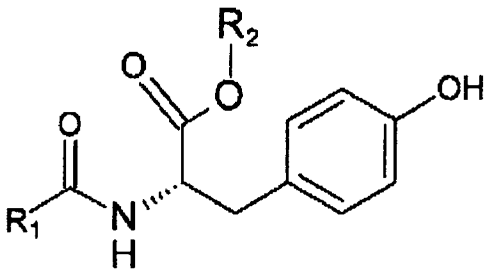
[wherein, $R_2$ is hydrogen, methyl, or ethyl], and



(III)

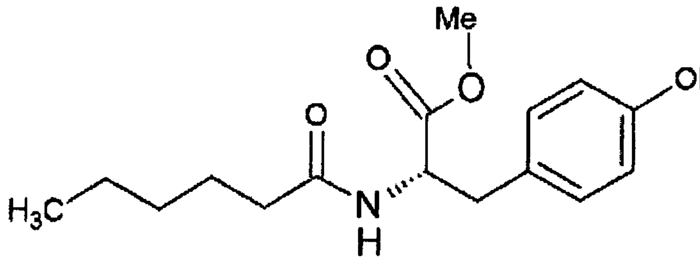
[wherein, $R_1$ is C1~C17 straight chain and branched alkyl, phenyl, or benzyl].

5. A composition for promoting secretion of antimicrobial peptides *in vivo,* the composition comprising a compound of Chemical Formula (I) or Chemical Formula (Ia) below as an active ingredient:



(I)

,



(Ia)

6. The composition of claim 5, wherein the compound as an active ingredient is contained at 0.001-90 wt%.

7. The composition for an external preparation of claim 5 or 6, wherein the composition is a liquid phase, an emulsion phase, a suspension phase, a cream phase, an ointment phase, a gel phase, a jelly phase, or a spray phase, as an external preparation.

8. The composition for systemic administration of claim 5 or 6, wherein the composition is a tablet for oral administration, a liquid, a powder, or an injection, as a composition for systemic administration.

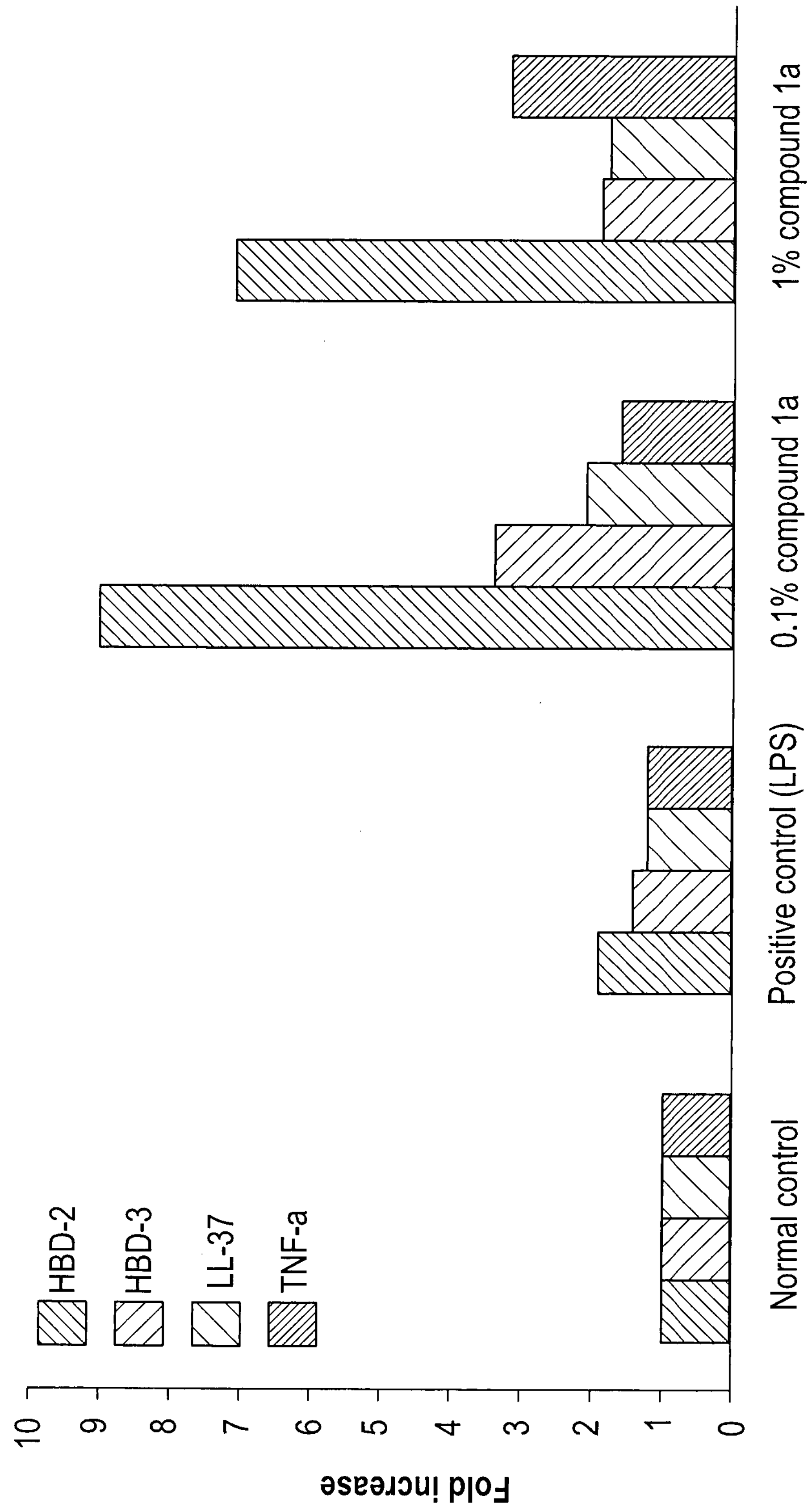

Fold increase
0
1
2
3
4
5
6
7
8
9
10
HBD-2
HBD-3
LL-37
TNF-a
Normal control
Positive control (LPS)
0.1% compound 1a
1% compound 1a

FIG. 1

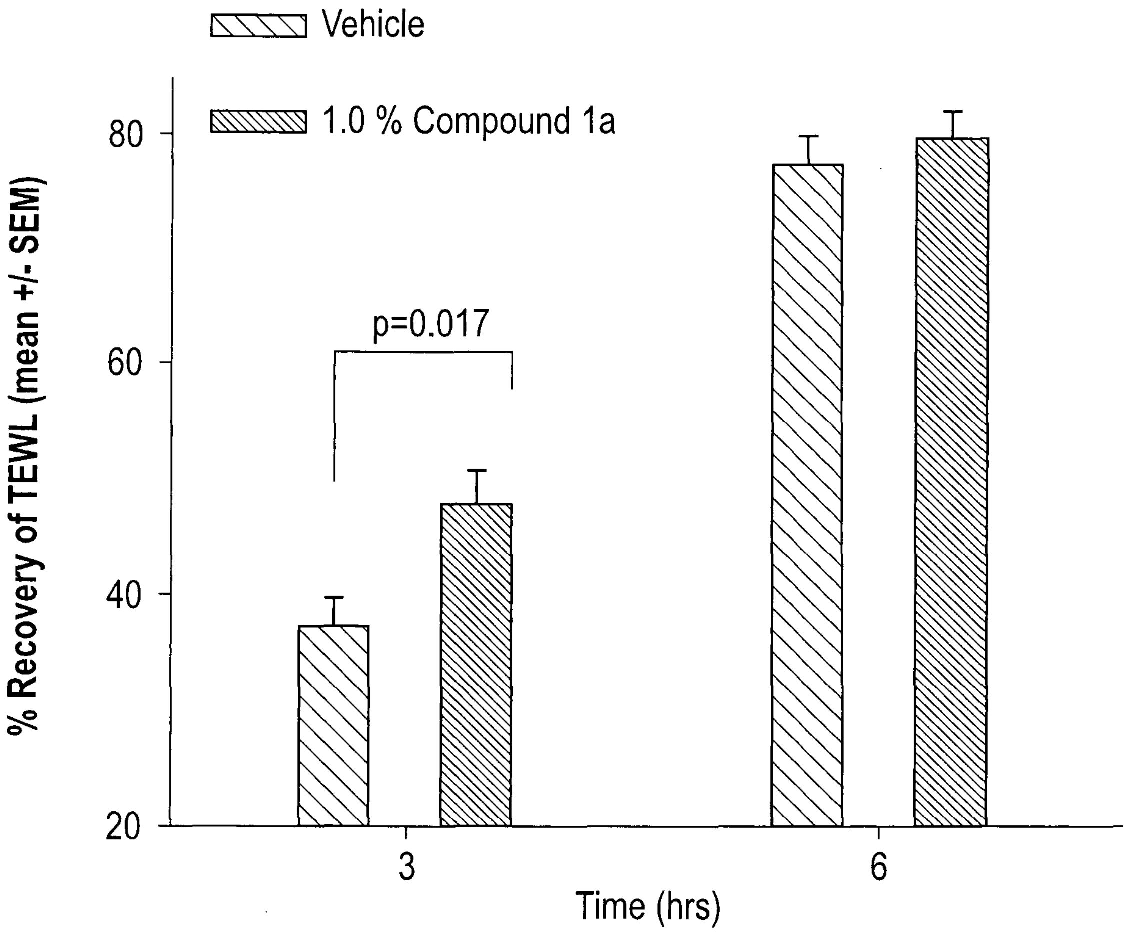

Vehicle
1.0 % Compound 1a
% Recovery of TEWL (mean +/- SEM)
80
60
40
20
p=0.017
3
6
Time (hrs)

FIG. 2

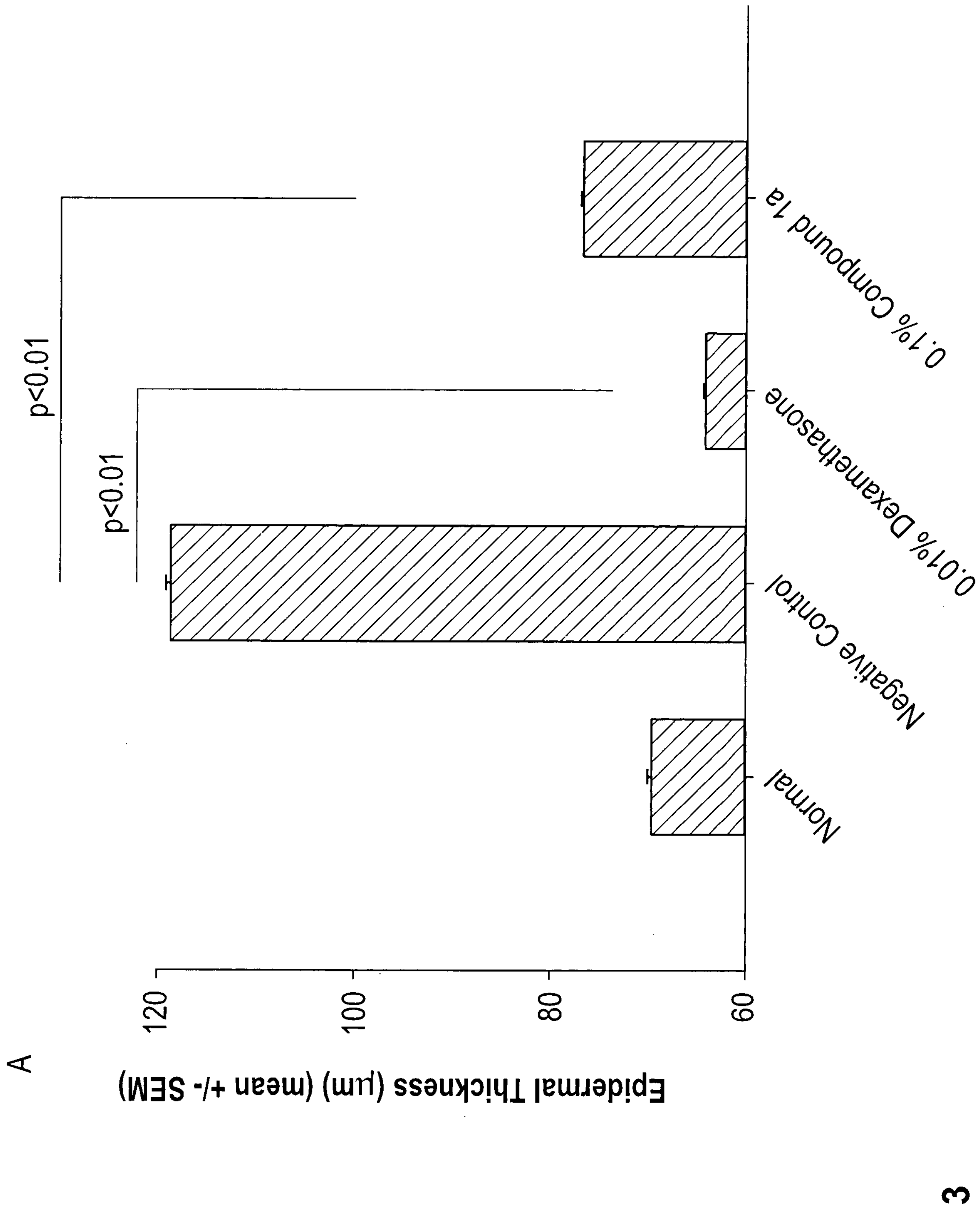
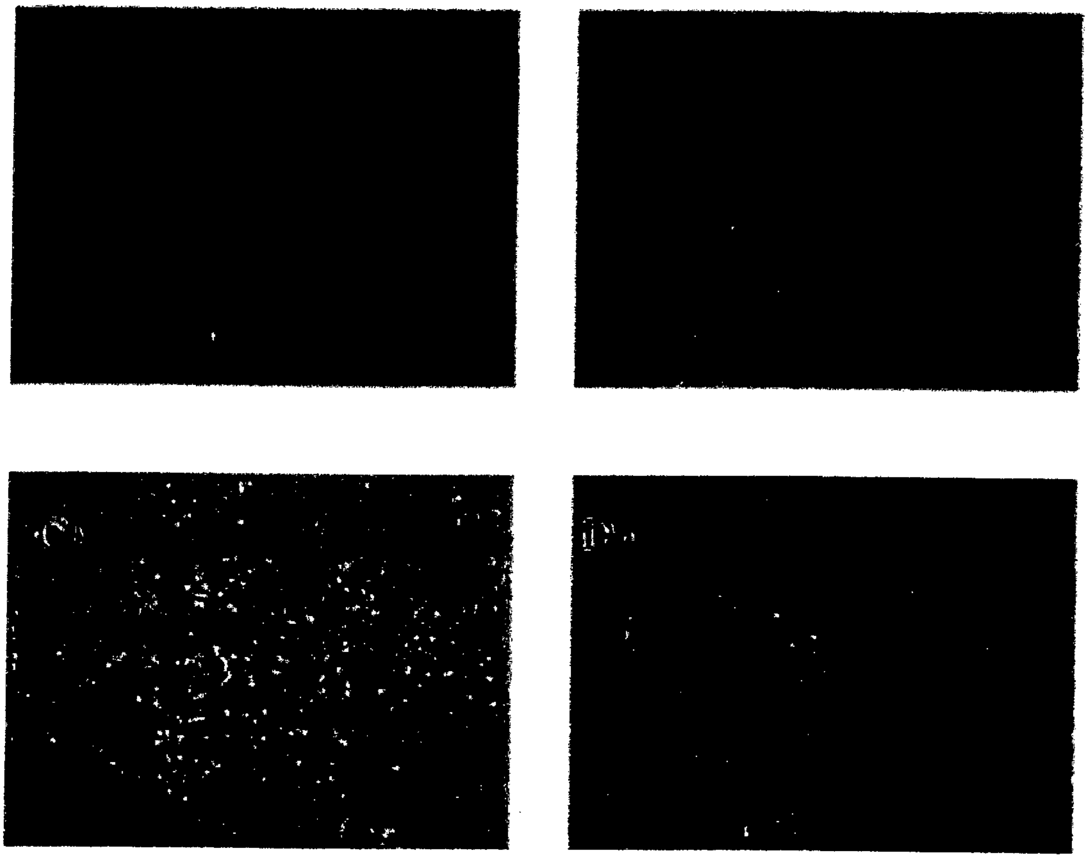

A
Epidermal Thickness (μm) (mean +/- SEM)
120
100
80
60
p<0.01
p<0.01
Normal
Negative Control
0.01% Dexamethasone
0.1% Compound 1a

FIG. 3

FIG. 4



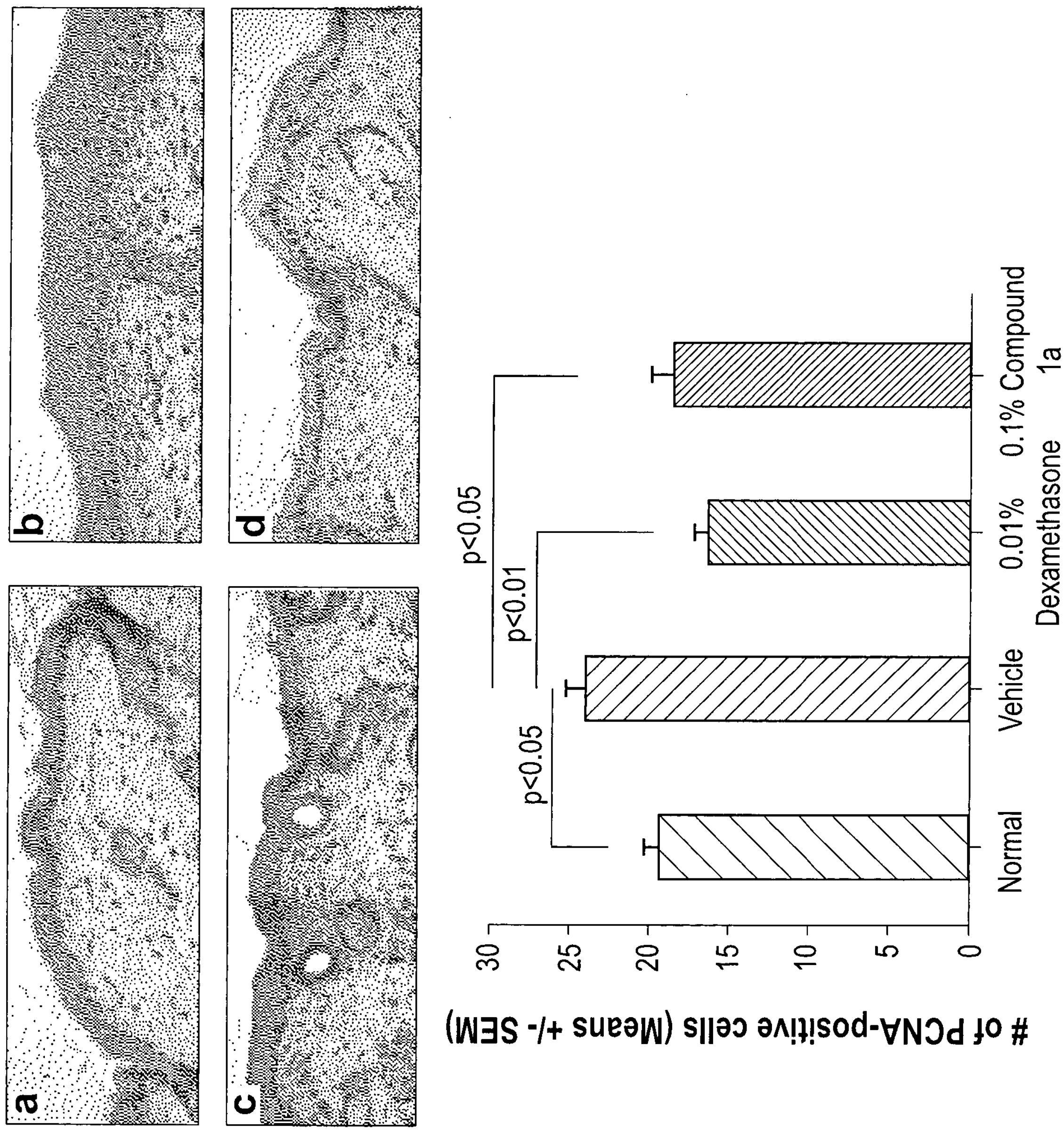

a
b
c
d
# of PCNA-positive cells (Means +/- SEM)
0
5
10
15
20
25
30
p<0.05
p<0.01
p<0.05
Normal
Vehicle
0.01% Dexamethasone
0.1% Compound 1a

FIG. 5

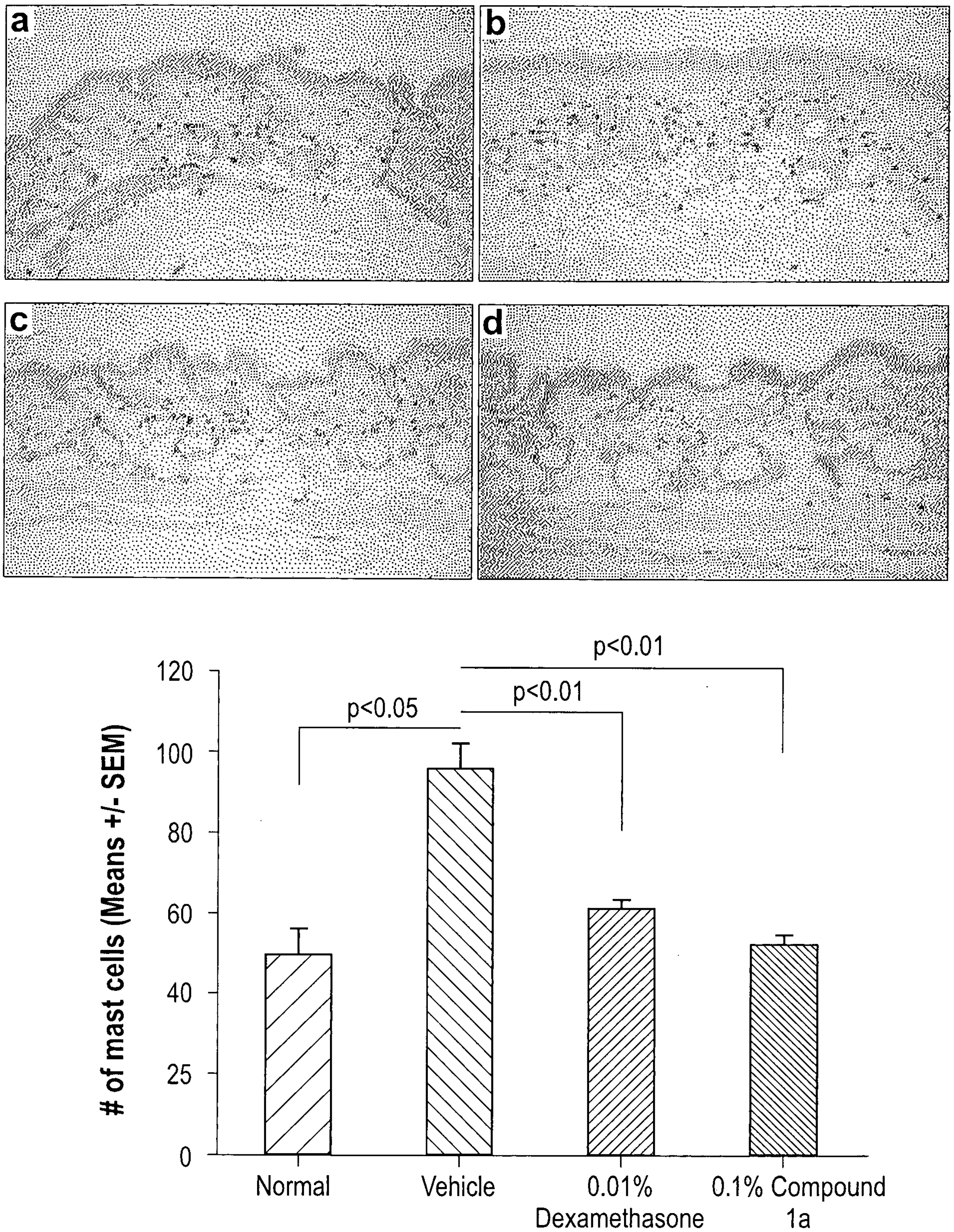
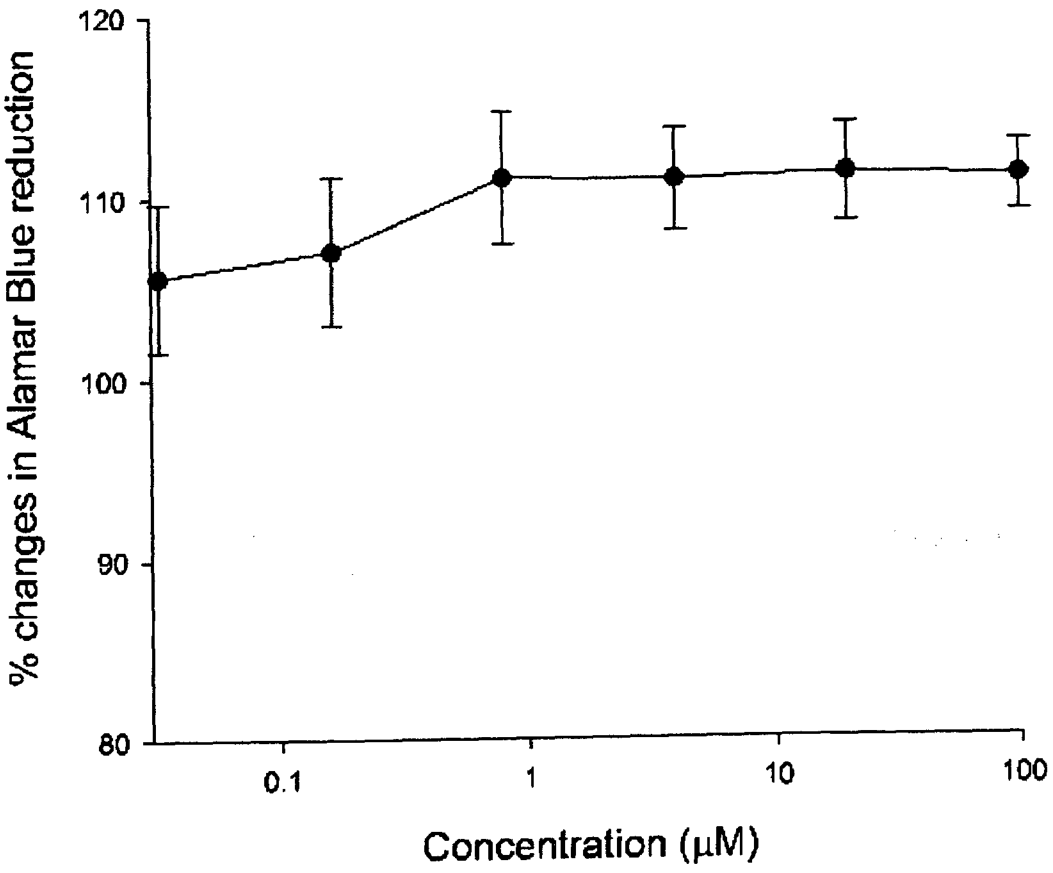

a
b
c
d
p<0.01
p<0.05
p<0.01
# of mast cells (Means +/- SEM)
120
100
80
60
40
25
0
Normal
Vehicle
0.01% Dexamethasone
0.1% Compound 1a

FIG. 6

FIG. 7


120
110
100
90
80
% changes in Alamar Blue reduction
0.1
1
10
100
Concentration (µM)

## INTERNATIONAL SEARCH REPORT

International application No.
**PCT/KR2010/005759**

A. CLASSIFICATION OF SUBJECT MATTER

***C07C 233/46(2006.01)i, A61K 31/198(2006.01)i***

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07C 233/46

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Utility models and applications for Utility models: IPC as above
Japanese Utility models and applications for Utility models: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS (KIPO internal) & Keywords: TYROSINE, PHARMACEUTICAL COMPOSITION

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>A | DE 10-2008-031556 A1 (HENKEL A. -G. & CO.) (30 April 2009)<br>See [0015] and pages 1-4 | 1-2,3-4<br>5-8 |
| X<br><br>A | MHASKAR, S.Y. et al., Synthesis of N-Acyl Amino Acids and Correlation of Structure with Surfactant Properties of Their Sodium Salt, JAOCS. Vol. 67, no. 12, pp 1015-1019 (31 December 1990)<br>See page 1016 | 1-2,3-4<br><br>5-8 |
| X<br><br>A | GEORGIADES, S. et al., Synthetic libraries of tyrosine-derived bacterial metabolites, Bioorganic & Medicinal Chemistry Letters 18, pp. 3117-3121 (22 October 2007)<br>See page 3117 | 1-2,3-4<br><br>5-8 |
| X<br><br>A | BRADY, S. F., Long-Chain N-Acyltyrosine Synthases from Environmental DNA, Applied and Environmental Microbiology, 70(11), pp. 6865-6870 (30 November 2004)<br>See page 6868 | 1-2,3-4<br><br>5-8 |

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 03 JUNE 2011 (03.06.2011) | **07 JUNE 2011 (07.06.2011)** |
| Name and mailing address of the ISA/KR<br>Korean Intellectual Property Office<br>Government Complex-Daejeon, 139 Seonsa-ro, Daejeon 302-701, Republic of Korea<br>Facsimile No. 82-42-472-7140 | Authorized officer<br><br>Telephone No. |

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
**PCT/KR2010/005759**

| Patent document cited in search report | Publication date | Patent family member | Publication date |
|---|---|---|---|
| NONE | | | |

Form PCT/ISA/210 (patent family annex) (July 2009)

## REFERENCES CITED IN THE DESCRIPTION



### Patent documents cited in the description


- KR 1020060076775 **[0007]**
- WO 0168085 A **[0007]**


### Non-patent literature cited in the description


- **BRAFF MH ; BARDAN A ; NIZET V et al.** Cutaneous defense mechanisms by antimicrobial peptides. *J Invest Dermatol,* 2005, vol. 125, 9 **[0004]**
- **NIYONSABA F ; USHIO H ; NAKANO N et al.** Antimicrobial peptides human β -defensins promote epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J Invest Dermatol,* 2007, vol. 127, 594 **[0005]**
- **ONG PY et al.** Endogenous Antimicrobial Peptides and Skin Infections in Atopic Dermatitis. *The England Journal of Medicine,* 2002, vol. 347, 1151-1160 **[0005]**
- **KIM JE ; KIM BJ ; JEONG MS et al.** Expression and Modulation of LL-37 in Normal Human Keratinocytes HaCaT Cells and Inflammatory Skin Diseases. *J Korean Med Sci,* 2005, vol. 20, 649 **[0094]**
- **PERNET I ; REYMERMIER C ; GUEZENNEC A et al.** Calcium triggers beta-defensin (hBD-2 and hBD-3) and chemokine macrophage inflammatory protein-3 alpha (MIP-3alpha/CCL20) expression in monolayers of activated human keratinocytes. *Exp Dermatol,* 2003, vol. 12, 755 **[0094]**
- **MAN M-Q ; HATANO Y ; LEE SH et al.** Characterization of a hepten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges. *J Invest Dermatol,* 2008, vol. 128, 79-86 **[0112]**